

B11

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
17 October 2002 (17.10.2002)

PCT

(10) International Publication Number
WO 02/081722 A2

(51) International Patent Classification⁷: C12P 13/08 //
(C12P 13/08, C12R 1:19)

(21) International Application Number: PCT/EP02/02421

(22) International Filing Date: 6 March 2002 (06.03.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
101 16 518.8 3 April 2001 (03.04.2001) DE

CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(71) Applicant: DEGUSSA AG [DE/DE]; Bennigsenplatz 1, 40474 Düsseldorf (DE).

(72) Inventors: RIEPING, Mechthild; Mönkebergstrasse 1, 33619 Bielefeld (DE). HERMANN, Thomas; Zirkonstrasse 8, 33739 Bielefeld (DE).

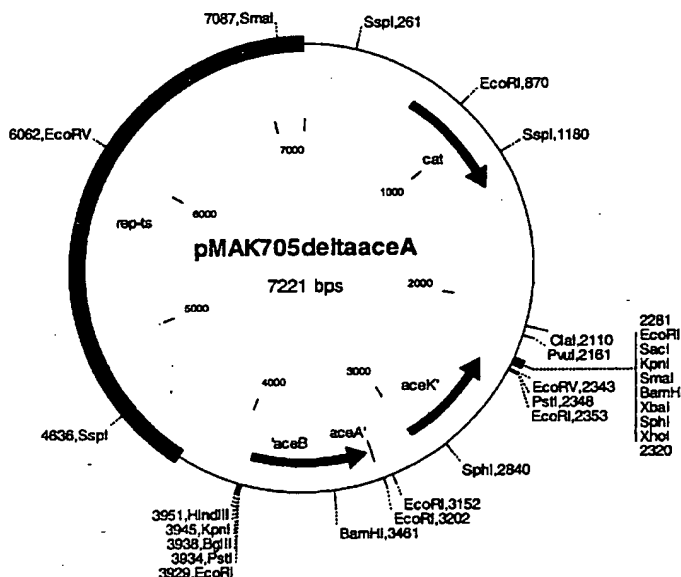
Published:

- without international search report and to be republished upon receipt of that report
- with (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: PROCESS FOR THE PRODUCTION OF L-AMINO ACIDS USING STRAINS OF THE FAMILY ENTEROBACTERIACEAE THAT CONTAIN AN ATTENUATED ACEA GENE



(57) Abstract: The invention relates to a process for the production of L-amino acids, in particular L-threonine, in which the following steps are carried out: a) fermentation of the microorganisms of the family Enterobacteriaceae producing the desired L-amino acid, in which the aceA gene or nucleotide sequences coding therefor are attenuated, in particular are switched off; b) enrichment of the L-amino acid in the medium or in the cells of the bacteria; and c) isolation of the L-amino acid.

WO 02/081722 A2

**Process for the Production of L-Amino Acids using
Strains of the Family Enterobacteriaceae that contain
an Attenuated aceA Gene**

Field of the Invention

- 5 The present invention relates to a process for the enzymatic production of L-amino acids, in particular L-threonine, using strains of the family Enterobacteriaceae in which the aceA gene is attenuated.

Prior Art

- 10 L-amino acids, in particular L-threonine, are used in human medicine and in the pharmaceutical industry, in the foodstuffs industry, and most especially in animal nutrition.

- It is known to produce L-amino acids by fermentation of
15 strains of Enterobacteriaceae, in particular Escherichia coli (E. coli) and Serratia marcescens. On account of their great importance efforts are constantly being made to improve processes for producing the latter. Process improvements may relate to fermentation technology
20 measures, such as for example stirring and provision of oxygen, or the composition of the nutrient media, such as for example the sugar concentration during the fermentation, or the working-up to the product form, for example by ion exchange chromatography, or the intrinsic
25 performance properties of the microorganism itself.

- Methods comprising mutagenesis, selection and mutant choice are employed in order to improve the performance properties of these microorganisms. In this way strains are obtained that are resistant to antimetabolites, such as for example
30 the threonine analogue α -amino- β -hydroxyvaleric acid (AHV) or are auxotrophic for regulatorily important metabolites, and that produce L-amino acids such as for example L-threonine.

Methods of recombinant DNA technology have also been used for some years in order to improve strains of the family Enterobacteriaceae producing L-amino acids, by amplifying individual amino acid biosynthesis genes and investigating
5 their effect on production.

Object of the Invention

The object of the invention is to provide new measures for the improved enzymatic production of L-amino acids, in particular L-threonine.

10 Summary of the Invention

The invention provides a process for the enzymatic production of L-amino acids, in particular L-threonine, using microorganisms of the family Enterobacteriaceae that in particular already produce L-amino acids and in which
15 the nucleotide sequence coding for the aceA gene is attenuated.

Detailed Description of the Invention

Where L-amino acids or amino acids are mentioned hereinafter, this is understood to mean one or more amino
20 acids including their salts, selected from the group comprising L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and
25 L-arginine. L-threonine is particularly preferred.

The term "attenuation" describes in this connection the reduction or switching off of the intracellular activity of one or more enzymes (proteins) in a microorganism that are coded by the corresponding DNA, by using for example a weak
30 promoter or a gene or allele that codes for a corresponding enzyme with a low activity and/or that inactivates the

corresponding enzyme (protein) or gene, and optionally combining these measures.

By means of these attenuation measures the activity or concentration of the corresponding protein is generally
5 reduced to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild type protein, or the activity or concentration of the protein in the initial microorganism.

The process is characterized in that the following steps
10 are carried out:

- a) fermentation of microorganisms of the family Enterobacteriaceae in which the aceA gene is attenuated,
- 15 b) enrichment of the corresponding L-amino acid in the medium or in the cells of the microorganisms of the family Enterobacteriaceae, and
- c) isolation of the desired L-amino acid, in which optionally constituents of the fermentation broth and/or the biomass in its entirety or portions
20 thereof remain in the product.

The microorganisms that are the subject of the present invention can produce L-amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, optionally starch, optionally cellulose or from glycerol and ethanol. The
25 microorganisms are members of the family Enterobacteriaceae selected from the genera *Escherichia*, *Erwinia*, *Providencia* and *Serratia*. The genera *Escherichia* and *Serratia* are preferred. In the case of the genus *Escherichia* the species *Escherichia coli* may in particular be mentioned,
30 and in the case of the genus *Serratia* the species *Serratia marcescens* may in particular be mentioned.

Suitable strains of the genus *Escherichia*, in particular those of the species *Escherichia coli*, that produce in particular L-threonine, include for example:

- Escherichia coli TF427
- 5 Escherichia coli H4578
- Escherichia coli KY10935
- Escherichia coli VNIIgenetika MG442
- Escherichia coli VNIIgenetika M1
- Escherichia coli VNIIgenetika 472T23
- 10 Escherichia coli BKIIM B-3996
- Escherichia coli kat 13
- Escherichia coli KCCM-10132

Suitable strains of the genus *Serratia*, in particular of the species *Serratia marcescens*, that produce L-threonine
15 include for example:

- Serratia marcescens* HNr21
- Serratia marcescens* TLR156
- Serratia marcescens* T2000

- Strains of the family of Enterobacteriaceae producing
- 20 L-threonine preferably have, *inter alia*, one or more of the genetic or phenotype features selected from the following group: resistance to α -amino- β -hydroxyvaleric acid, resistance to thialysine, resistance to ethionine, resistance to α -methylserine, resistance to diaminosuccinic
 - 25 acid, resistance to α -aminobutyric acid, resistance to borrelidin, resistance to rifampicin, resistance to valine analogues such as for example valine hydroxamate, resistance to purine analogues such as for example 6-dimethylaminopurine, need for L-methionine, optionally
 - 30 partial and compensable need for L-isoleucine, need for meso-diaminopimelic acid, auxotrophy with regard to threonine-containing dipeptides, resistance to L-threonine, resistance to L-homoserine, resistance to L-lysine, resistance to L-methionine, resistance to L-glutamic acid.

resistance to L-aspartate, resistance to L-leucine,
resistance to L-phenylalanine, resistance to L-serine,
resistance to L-cysteine, resistance to L-valine,
sensitivity to fluoropyruvate, defective threonine
5 dehydrogenase, optionally ability to utilize sucrose,
enhancement of the threonine operon, enhancement of
homoserine dehydrogenase, I-aspartate kinase I, preferably
of the feedback-resistant form, enhancement of homoserine
kinase, enhancement of threonine synthase, enhancement of
10 aspartate kinase, optionally of the feedback-resistant
form, enhancement of aspartate semialdehyde dehydrogenase,
enhancement of phosphoenol pyruvate carboxylase, optionally
of the feedback-resistant form, enhancement of phosphoenol
pyruvate synthase, enhancement of transhydrogenase,
15 enhancement of the RhtB gene product, enhancement of the
RhtC gene product, enhancement of the YfiK gene product,
enhancement of a pyruvate carboxylase, and attenuation of
acetic acid formation.

It has now been found that microorganisms of the family
20 Enterobacteriaceae after attenuation, in particular after
switching off the aceA gene, produce L-amino acids, in
particular L-threonine, in an improved way.

The nucleotide sequences of the Escherichia coli genes
belong to the prior art and may also be obtained from the
25 genome sequence of Escherichia coli published by Blattner
et al. (Science 277, 1453 - 1462 (1997)).

The aceA gene is described *inter alia* by the following
data:

Designation:	Isocitrate lyase
30 EC-No.:	4.1.3.1
Reference:	Matsuoko and McFadden; Journal of Bacteriology 170, 4528-4536 (1988)
Accession No.:	AE000474

Apart from the described *aceA* gene, alleles of the gene may be used that result from the degeneracy of the genetic code or from functionally neutral sense mutations, the activity of the protein not being substantially altered.

- 5 In order to achieve an attenuation the expression of the gene or the catalytic properties of the enzyme proteins may for example be reduced or switched off. Optionally both measures may be combined.

The gene expression may be reduced by suitable culture
10 conditions, by genetic alteration (mutation) of the signal structures of the gene expression, or also by antisense-RNA techniques. . Signal structures of the gene expression are for example repressor genes, activator genes, operators, promoters, attenuators, ribosome-binding sites, the start
15 codon and terminators. The person skilled in the art may find relevant information in, *inter alia*, articles by Jensen and Hammer (Biotechnology and Bioengineering 58: 191-195 (1998)), by Carrier and Keasling (Biotechnology Progress 15, 58-64 (1999), Franch and Gerdes (Current
20 Opinion in Microbiology 3, 159-164 (2000)) and in known textbooks of genetics and molecular biology, such as for example the textbook by Knippers ("Molekulare Genetik", 6th Edition, Georg Thieme Verlag, Stuttgart, Germany, 1995) or that by Winnacker ("Gene and Klone", VCH
25 Verlagsgesellschaft, Weinheim, Germany, 1990).

Mutations that lead to a change or reduction of the catalytic properties of enzyme proteins are known from the prior art. As examples there may be mentioned the work by Qiu and Goodman (Journal of Biological Chemistry 272: 8611-
30 8617 (1997)), Yano et al. (Proceedings of the National Academy of Sciences, USA 95, 5511-5515 (1998), Wentz and Schachmann (Journal of Biological Chemistry 266, 20833-20839 (1991). Detailed information may be obtained from known textbooks on genetics and molecular biology, such as

for example that by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

Suitable mutations include transitions, transversions, insertions and deletions. Depending on the action of the amino acid exchange on the enzyme activity, one speaks of missense mutations or nonsense mutations. Insertions or deletions of at least one base pair in a gene lead to frame shift mutations, which in turn lead to the incorporation of false amino acids or the premature termination of a translation. If as a result of the mutation a stop codon is formed in the coding region, this also leads to a premature termination of the translation. Deletions of several codons typically lead to a complete disruption of the enzyme activity. Details regarding the production of such mutations belong to the prior art and may be obtained from known textbooks on genetics and molecular biology, such as for example the textbook by Knippers ("Molekulare Genetik", 6th Edition, Georg Thieme Verlag, Stuttgart, Germany, 1995), that by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990) or that by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

Suitable mutations in the genes such as for example deletion mutations may be incorporated by gene and/or allele exchange in suitable strains.

A conventional method is the method of gene exchange by means of a conditionally replicating pSC101 derivate pMAK705 described by Hamilton et al. (Journal of Bacteriology 171, 4617 - 4622 (1989)). Other methods described in the prior art, such as for example that of Martinez-Morales et al. (Journal of Bacteriology 181, 7143-7148 (1999)) or that of Boyd et al. (Journal of Bacteriology 182, 842-847 (2000)) may likewise be used.

It is also possible to transfer mutations in the respective genes or mutations relating to the expression of the relevant genes, by conjugation or transduction into various strains.

- 5 Furthermore for the production of L-amino acids, in particular L-threonine, using strains of the family Enterobacteriaceae it may be advantageous in addition to the attenuation of the aceA gene also to enhance one or more enzymes of the known threonine biosynthesis pathway or
10 enzymes of anaplerotic metabolism or enzymes for the production of reduced nicotinamide-adenine-dinucleotide phosphate.

The term "enhancement" describes in this connection the raising of the intracellular activity of one or more
15 enzymes or proteins in a microorganism that are coded by the corresponding DNA, by for example increasing the number of copies of the gene or genes, using a strong promoter or a gene that codes for a corresponding enzyme or protein having a high activity, and optionally by combining these
20 measures.

By means of the enhancement measures, in particular overexpression, the activity or concentration of the corresponding protein is in general raised by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, at
25 most up to 1000% or 2000% referred to that of the wild type protein and/or the activity or concentration of the protein in the initial microorganism.

Thus, one or more of the genes selected from the following group may for example be simultaneously enhanced, in
30 particular overexpressed:

- the thrABC operon coding for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase (US-A-4,273,763).

- the pyc gene coding for pyruvate carboxylase (DE-A-19 831 609),
- the pps gene coding for phosphoenol pyruvate synthase (Molecular and General Genetics 231:332 (1992)),
- 5 • the ppc gene coding for phosphoenol pyruvate carboxylase (Gene 31:279-283 (1984)),
- the genes pntA and pntB coding for transhydrogenase (European Journal of Biochemistry 158:647-653 (1986)),
- the gene rhtB imparting homoserine resistance (EP-A-0 994 190),
- 10 • the mgo gene coding for malate:quinone oxidoreductase (DE 100 348 33.5),
- the gene rhtC imparting threonine resistance (EP-A-1 013 765), and
- 15 • the thrE gene of Corynebacterium glutamicum coding for threonine export (DE 100 264 94.8).

The use of endogenous genes is in general preferred. The term "endogenous genes" or "endogenous nucleotide sequences" is understood to mean the genes or nucleotide sequences
20 present in the population of a species.

Furthermore for the production of L-amino acids, in particular L-threonine, it may be advantageous in addition to the attenuation of the aceA gene also to attenuate, in particular to switch off or reduce the expression of one or
25 more of the genes selected from the following group:

- the tdh gene coding for threonine dehydrogenase (Ravnikar and Somerville, Journal of Bacteriology 169, 4716-4721 (1987)),

- the mdh gene coding for malate dehydrogenase (E.C. 1.1.1.37) (Vogel et al., Archives in Microbiology 149, 36-42 (1987)),
- the gene product of the open reading frame (orf) yjfa
5 (Accession Number AAC77180 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA),
- the gene product of the open reading frame (orf) ytfP (Accession Number AAC77179 des National Center for Biotechnology Information (NCBI, Bethesda, MD, USA),
- 10 • the pckA gene coding for the enzyme phosphoenol pyruvate carboxykinase (Medina et al. (Journal of Bacteriology 172, 7151-7156 (1990)),
- the poxB gene coding for pyruvate oxidase (Grabau and Cronan (Nucleic Acids Research 14 (13), 5449-5460
15 (1986)),
- the dgsA gene coding for the regulator of the phosphotransferase system (Hosono et al., Bioscience, Biotechnology and Biochemistry 59, 256-251 (1995) and Accession No.: AE000255), and
- 20 • the fruR gene coding for the fructose repressor (Jahreis et al., Molecular and General Genetics 226, 332-336 (1991) and Accession No.: AE000118)

Furthermore for the production of L-amino acids, in particular L-threonine, it may be advantageous in addition
25 to the attenuation of the aceA gene also to switch off undesirable secondary reactions (Nakayama: "Breeding of Amino Acid Producing Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

30 The microorganisms produced according to the invention may be cultivated in a batch process (batch cultivation), in a

- fed batch process (feed process) or in a repeated fed batch process (repetitive feed process). A summary of known cultivation methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die
- 5 Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Störhas (Bioreaktoren and periphere Einrichtungen (Vieweg Verlag, Brunswick /Wiesbaden, 1994)).

The culture medium to be used must appropriately satisfy

10 the requirements of the respective strains. Descriptions of culture media of various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

- 15 As carbon sources, sugars and carbohydrates such as for example glucose, sucrose, lactose, fructose, maltose, molasses, starch and optionally cellulose, oils and fats such as for example soya bean oil, sunflower oil, groundnut oil and coconut oil, fatty acids such as for example
- 20 palmitic acid, stearic acid and linoleic acid, alcohols such as for example glycerol and ethanol, and organic acids such as for example acetic acid, may be used. These substances may be used individually or as a mixture.

As nitrogen source, organic nitrogen-containing compounds

25 such as peptones, yeast extract, meat extract, malt extract, maize starch water, soya bean flour and urea or inorganic compounds such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate may be used. The nitrogen sources may be

30 used individually or as a mixture.

As phosphorus source, phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts may be used. The

soluble sodium salts of phosphoric acid may be used.

- such as for example magnesium sulfate or iron sulfate, that are necessary for growth. Finally, essential growth promoters such as amino acids and vitamins may be used in addition to the aforementioned substances. Apart from
- 5 these, suitable precursors may be added to the culture medium. The aforementioned starting substances may be added to the culture in the form of a single batch or may be metered in in an appropriate manner during the cultivation.
- 10 In order to regulate the pH of the culture basic compounds such as sodium hydroxide, potassium hydroxide, ammonia or ammonia water, or acidic compounds such as phosphoric acid or sulfuric acid are used as appropriate. In order to control foam formation antifoaming agents such as for
- 15 example fatty acid polyglycol esters may be used. In order to maintain the stability of plasmids, suitable selectively acting substances, for example antibiotics, may be added to the medium. In order to maintain aerobic conditions, oxygen or oxygen-containing gas mixtures such as for
- 20 example air are fed into the culture. The temperature of the culture is normally 25°C to 45°C, and preferably 30°C to 40°C. Cultivation is continued until a maximum amount of L-amino acids (or L-threonine) has been formed. This target is normally achieved within 10 hours to 160 hours.
- 25 The L-amino acids may be analyzed by anion exchange chromatography followed by ninhydrin derivation, as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190), or by reversed phase HPLC, as described by Lindroth et al. (Analytical Chemistry (1979) 51: 1167-
- 30 1174).

The process according to the invention can be used for the enzymatic production of L-amino acids, such as for example L-threonine, L-isoleucine, L-valine, L-methionine, L-homoserine and L-lysine, in particular L-threonine.

A pure culture of the Escherichia coli K-12 strain DH5 α /pMAK705 was filed as DSM 13720 on 08 September 2000 at the German Collection for Microorganisms and Cell Cultures (DSMZ, Brunswick, Germany) according to the Budapest
 5 Convention.

The present invention is described in more detail hereinafter with the aid of examples of implementation.

The isolation of plasmid DNA from Escherichia coli as well as all techniques for the restriction, Klenow treatment and
 10 alkaline phosphatase treatment are carried out according to Sambrook et al. (Molecular Cloning - A Laboratory Manual (1989) Cold-Spring Harbor Laboratory Press). The transformation of Escherichia coli is, unless otherwise described, carried out according to Chung et al.
 15 (Proceedings of the National Academy of Sciences of the United States of America, USA (1989) 86: 2172-2175).

The incubation temperature in the production of strains and transformants is 37°C. In the gene exchange process according to Hamilton et al., temperatures of 30°C and 44°C
 20 are used.

Example 1

Construction of the deletion mutation of the aceA gene.

Parts of the gene regions lying upstream and downstream of the aceA gene are amplified from Escherichia coli K12 using
 25 the polymerase chain reaction (PCR) as well as synthetic oligonucleotides. Starting from the nucleotide sequence of the aceBAK operon in E. coli K12 MG1655 DNA (SEQ ID No. 1) the following PCR primers are synthesized (MWG Biotech, Ebersberg, Germany):

30 aceA'5'-1: 5' - ATGCTTACTCACGCCTGTTG - 3' (SEQ ID No. 3)

aceA'5'-2: 5' - CAGTCTTACGTCGCTGTTG - 3' (SEQ ID No. 4)

aceA'3'-1: 5' - CAACAACAACCGTTGCTGAC - 3' (SEQ ID No. 5)

aceA'3'-2: 5' - CAGTTCGTTCCGCCACCTGTA - 3' (SEQ ID No. 6)

The chromosomal E. coli K12 MG1655 DNA used for the PCR is isolated according to the manufacturer's instructions using

5 "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany). A ca. 700 bp large DNA fragment from the region lying upstream of the aceA gene (designated 'aceB') and a ca. 800 bp large DNA fragment from the region lying downstream of the aceA gene (designated aceK') can be amplified with

10 the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A guide to methods and applications, Academic Press) with Taq DNA polymerase (Gibco-BRL, Eggenstein, Germany). The PCR products are ligated according to the manufacturer's instructions in

15 each case with the vector pCR2.1TOPO (TOPO TA Cloning Kit, Invitrogen, Groningen, Netherlands) and transformed in the E. coli strain TOP10F'. The selection of plasmid-carrying cells is carried out on LB agar to which 50 µg/ml of ampicillin has been added. After the plasmid DNA isolation

20 the vector pCR2.1TOPO'aceB is cleaved with the restriction enzymes EcoRV and SpeI, and the 'aceB' fragment after separation in 0.8% agarose gel is isolated using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). After the plasmid DNA isolation the vector pCR2.1TOPOaceK'

25 is cleaved with the enzymes Ecl136II and SpeI and ligated with the isolated 'aceB' fragment. The E. coli strain DH5α is transformed with the ligation batch and plasmid-carrying cells are selected on LB agar to which 50 µg/ml of ampicillin has been added. After the plasmid DNA

30 isolation, those plasmids in which the mutagenic DNA sequence shown in SEQ ID No. 7 is present in cloned form are detected by control cleavage with the enzymes HindIII and XbaI. One of the plasmids is designated pCR2.1TOPOΔaceA.

Example 2

Construction of the exchange vector pMAK705 Δ aceA

The aceBAK allele described in Example 1 is isolated from the vector pCR2.1TOPO Δ aceA after restriction with the
5 enzymes HindIII and XbaI and separation in 0.8% agarose gel, and is ligated with the plasmid pMAK705 (Hamilton et al. (1989) Journal of Bacteriology 171, 4617 - 4622), that had been digested with the enzymes HindIII and XbaI. The ligation batch is transformed in DH5 α and plasmid-carrying
10 cells are selected on LB agar to which 20 μ g/ml of chloramphenicol have been added. The successful cloning is detected after plasmid DNA isolation and cleavage with the enzymes BamHI, KpnI, SphI, SpeI and PstI. The resultant exchange vector pMAK705 Δ aceA (= pMAK705deltaaceA) is shown
15 in Fig. 1.

Example 3

Site-specific mutagenesis of the aceA gene in the E. coli strain MG442

The E. coli strain MG442 producing L-threonine is described
20 in patent specification US-A- 4,278,765 and is filed as CMIM B-1628 at the Russian National Collection for Industrial Microorganisms (VKPM, Moscow, Russia).

For the exchange of the chromosomal aceA gene by the plasmid-coded deletion construct, MG442 is transformed with
25 the plasmid pMAK705 Δ aceA. The gene exchange is carried out by the selection process described by Hamilton et al.

(1989) Journal of Bacteriology 171, 4617 - 4622) and is verified by standard PCR methods (Innis et al. (1990) PCR Protocols. A guide to methods and applications, Academic
30 Press) with the following oligonucleotide primers:

aceA'5'-1: 5' - ATGCTTACTCACGCCTGTTG - 3' (SEQ ID No. 3)

aceA'3'-2: 5' - CAGTTCGTTTCGCCACCTGTA - 3' (SEQ ID No. 6)

The resultant strain is designated MG442ΔaceA.

Example 4

Production of L-threonine using the strain MG442ΔaceA

- 5 MG442ΔaceA is cultivated on minimal medium having the following composition: 3.5 g/l $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 1.5 g/l KH_2PO_4 , 1 g/l NH_4Cl , 0.1 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g/l glucose and 20 g/l agar. The formation of L-threonine is checked in batch cultures of 10 ml that are contained in 100 ml Erlenmeyer
- 10 flasks. For this, 10 ml of preculture medium of the following composition: 2 g/l yeast extract, 10 g/l $(\text{NH}_4)_2\text{SO}_4$, 1 g/l KH_2PO_4 , 0.5 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 15 g/l CaCO_3 , 20 g/l glucose are inoculated and incubated for 16 hours at 37°C and 180 rpm in an ESR incubator from Kühner AG
- 15 (Birsfelden, Switzerland). 250 µl of this preculture are reinoculated in 10 ml of production medium (25 g/l $(\text{NH}_4)_2\text{SO}_4$, 2 g/l KH_2PO_4 , 1 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03 g/l $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.018 g/l $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 30 g/l CaCO_3 and 20 g/l glucose) and incubated for 48 hours at 37°C. After incubation the
- 20 optical density (OD) of the culture suspension is measured with an LP2W photometer from the Dr. Lange company (Dusseldorf, Germany) at a measurement wavelength of 660 nm.

- The concentration of formed L-threonine is then determined
- 25 in the sterile-filtered culture supernatant using an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column reaction with ninhydrin detection.

The result of the test is given in Table 1.

Table 1

Strain	OD (660 nm)	L-threonine g/l
MG442	6.0	1.5
MG442 Δ aceA	6.2	1.9

Brief Description of the Figure:

- Fig. 1: pMAK705 Δ aceA (= pMAK705deltaaceA)

5 Length data are given as approximate values. The abbreviations and acronyms used have the following meanings:

- cat: chloramphenicol resistance gene
- rep-ts: temperature-sensitive replication region of
10 the plasmid pSC101
- 'aceB: part of the 3' region of the aceB gene
- aceA': ATG start codon of the aceA gene
- aceK': part of the 5' region of the aceK gene

The abbreviations for the restriction enzymes have the
15 following meanings:

- BamHI: restriction endonuclease from *Bacillus* amyloliquefaciens
- BglII: restriction endonuclease from *Bacillus* globigii
- 20 • ClaI: restriction endonuclease from *Caryophanon latum*

• EcoRI: restriction endonuclease from *Escherichia coli*

- EcoRV: restriction endonuclease from *Escherichia coli*
- HindIII: restriction endonuclease from *Haemophilus influenzae*
- 5 • KpnI: restriction endonuclease from *Klebsiella pneumoniae*
- PstI: restriction endonuclease from *Providencia stuartii*
- PvuI: restriction endonuclease from *Proteus vulgaris*
- 10 • SacI: restriction endonuclease from *Streptomyces achromogenes*
- SalI: restriction endonuclease from *Streptomyces albus*
- SmaI: restriction endonuclease from *Serratia marcescens*
- 15 • SphI: restriction endonuclease from *Streptomyces phaeochromogenes*
- SspI: restriction endonuclease from *Sphaerotilus species*
- XbaI: restriction endonuclease from *Xanthomonas badrii*
- 20 • XhoI: restriction endonuclease from *Xanthomonas holcicola*

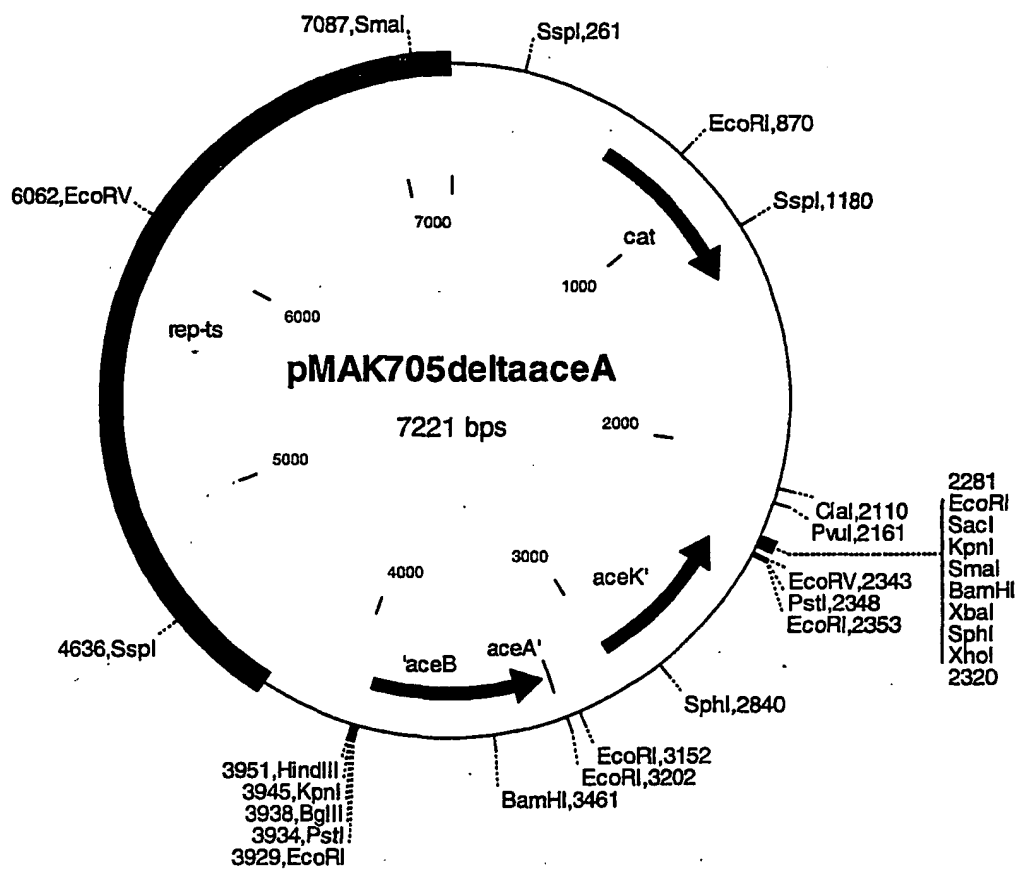
What is Claimed is:

1. Process for the production of L-amino acids, in particular L-threonine, wherein the following steps are carried out:
 - 5 a) fermentation of the microorganisms of the family Enterobacteriaceae producing the desired L-amino acid, in which the aceA gene or nucleotide sequences coding therefor are attenuated, in particular are switched off,
 - 10 b) enrichment of the L-amino acid in the medium or in the cells of the microorganisms, and
 - c) isolation of the L-amino acid, in which optionally constituents of the fermentation broth and/or the biomass in its entirety or portions
15 thereof remain in the product.
2. Process according to claim 1, wherein microorganisms are used in which in addition further genes of the biosynthesis pathway of the desired L-amino acid are enhanced.
- 20 3. Process according to claim 1, wherein microorganisms are used in which the metabolic pathways that reduce the formation of the desired L-amino acid are at least partially switched off.
4. Process according to claim 1, wherein the expression
25 of the polynucleotide(s) that codes/code for the aceA gene is attenuated, in particular is switched off.
5. Process according to claim 1, wherein the regulatory and/or catalytic properties of the polypeptide (enzyme protein) for which the polynucleotide aceA codes are
30 reduced.

6. Process according to claim 1, wherein, for the production of L-amino acids, microorganisms of the family Enterobacteriaceae are fermented in which at the same time one or more of the genes selected from the following group is enhanced, in particular overexpressed:
- 6.1 the thrABC operon coding for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase,
- 6.2 the pyc gene coding for pyruvate carboxylase,
- 6.3 the pps gene coding for phosphoenol pyruvate synthase,
- 6.4 the ppc gene coding for phosphoenol pyruvate carboxylase,
- 6.5 the genes pntA and pntB coding for transhydrogenase,
- 6.6 the gene rhtB imparting homoserine resistance,
- 6.7 the mqo gene coding for malate:quinone oxidoreductase,
- 6.8 the gene rhtC imparting threonine resistance, and
- 6.9 the thrE gene coding for threonine export.
7. Process according to claim 1, wherein, for the production of L-amino acids, microorganisms of the family Enterobacteriaceae are fermented in which at the same time one or more of the genes selected from the following group is attenuated, in particular switched off, or the expression is reduced:
- 7.1 the tdh gene coding for threonine dehydrogenase,
- 7.2 the adh gene coding for alcohol dehydrogenase,

- 7.3 the gene product of the open reading frame (orf)
yjfa,
- 7.4 the gene product of the open reading frame (orf)
ytfP,
- 5 7.5 the pckA gene coding for phosphoenol pyruvate
carboxykinase,
- 7.6 the poxB gene coding for pyruvate oxidase,
- 7.7 the dgsA gene coding for the regulator of the
phosphotransferase system, and
- 10 7.8 the fruR gene coding for the fructose repressor.

Fig. 1:



SEQUENCE LISTING

5 <110> Degussa AG
 <120> Process for the production of L-amino acids using strains of the family Enterobacteriaceae containing an attenuated aceA gene
 10 <130> 010131 BT
 <160> 7
 <170> PatentIn version 3.1
 15 <210> 1
 <211> 4855
 <212> DNA
 <213> Escherichia coli
 20 <220>
 <221> CDS
 <222> (1632)..(2936)
 <223> aceA gene
 25 <400> 1
 atgactgaac aggcaacaac aaccgatgaa ctggctttca caaggccgta tggcgagcag 60
 gagaagcaaa ttcttactgc cgaagcggta gaattttctga ctgagctggt gacgcatttt 120
 30 acgccacaac gcaataaact tctggcagcg cgcattcagc agcagcaaga tattgataac 180
 ggaacgttgc ctgattttat ttcggaaaca gcttccattc gcgatgctga ttggaaaatt 240
 cgcgggattc ctgcggaactt agaagaccgc cgcgtagaga taactggccc ggtagagcgc 300
 35 aagatggtga tcaacgcgct caacgccaat gtgaaagtct ttatggccga tttcgaagat 360
 tcaactggcac cagactggaa caaagtgatc gacgggcaaa ttaacctgcg tgatgcggtt 420
 40 aacggcacca tcagttacac caatgaagca ggcaaaattt accagctcaa gcccaatcca 480
 gcggttttga tttgtcgggt acgcggtctg cacttgccgg aaaaacatgt cacctggcgt 540
 ggtgaggcaa tccccggcag cctgtttgat tttgcgctct atttcttcca caactatcag 600
 45 gcactgttgg caaagggcag tggctccctat ttctatctgc cgaaaaccca gtcctggcag 660
 gaagcggcct ggtggagcga agtcttcagc tatgcagaag atcgctttaa tctgccgcgc 720
 50 ggcaccatca aggcgacgtt gctgattgaa acgctgcccg ccgtgttcca gatggatgaa 780
 atccttcacg cgctgcgtga ccatattgtt ggtctgaact gcggtcgttg ggattacatc 840
 ttcagctata tcaaaacgtt gaaaaactat cccgatcgcg tcctgccaga cagacaggca 900
 55 gtgacgatgg ataaaccatt cctgaatgct tactcacgcc tggtgattaa aacctgccat 960
 aaacgcggtg cttttgcgat gggcggcatg gcggcgttta ttccgagcaa agatgaagag 1020
 60 cacaataacc aggtgctcaa caaagtaaaa gcggataaat cgctggaagc caataacggt 1080
 cacgatggca catggatcgc tcaccaggc cttgaggaca cggcaatggc ggtattcaac 1140
 65 gaaatgctga gaaatgctga gaaatgctga gaaatgctga gaaatgctga gaaatgctga 1200

	actgccgatac agctgctggc accttgtgat ggtgaacgca ccgaagaagg tatgcgcgcc	1260
	aacattcgcg tggctgtgca gtacatcgaa gcgtggatct ctggcaacgg ctgtgtgccg	1320
5	atztatggcc tgatggaaga tgcggcgacg gctgaaattt cccgtacctc gatctggcag	1380
	tggtatccatc atcaaaaaac gttgagcaat ggcaaacggg tgaccaaagc cttgttccgc	1440
10	cagatgctgg gcgaagagat gaaagtcatt gccagcgaac tgggcgaaga acgtttctcc	1500
	caggggcggtt ttgacgatgc cgcacgcttg atggaacaga tcaccacttc cgatgagtta	1560
	attgatttcc tgaccctgcc aggctaccgc ctgttagcgt aaaccaccac ataactatgg	1620
15	agcatctgca c atg aaa acc cgt aca caa caa att gaa gaa tta cag aaa Met Lys Thr Arg Thr Gln Gln Ile Glu Glu Leu Gln Lys 1 5 10	1670
20	gag tgg act caa ccg cgt tgg gaa ggc att act cgc cca tac agt gcg Glu Trp Thr Gln Pro Arg Trp Glu Gly Ile Thr Arg Pro Tyr Ser Ala 15 20 25	1718
25	gaa gat gtg gtg aaa tta cgc ggt tca gtc aat cct gaa tgc acg ctg Glu Asp Val Val Lys Leu Arg Gly Ser Val Asn Pro Glu Cys Thr Leu 30 35 40 45	1766
30	gcg caa ctg ggc gca gcg aaa atg tgg cgt ctg ctg cac ggt gag tcg Ala Gln Leu Gly Ala Ala Lys Met Trp Arg Leu Leu His Gly Glu Ser 50 55 60	1814
35	aaa aaa ggc tac atc aac agc ctc ggc gca ctg act ggc ggt cag gcg Lys Lys Gly Tyr Ile Asn Ser Leu Gly Ala Leu Thr Gly Gly Gln Ala 65 70 75	1862
40	ctg caa cag gcg aaa gcg ggt att gaa gca gtc tat ctg tcg gga tgg Leu Gln Gln Ala Lys Ala Gly Ile Glu Ala Val Tyr Leu Ser Gly Trp 80 85 90	1910
45	cag gta gcg gcg gac gct aac ctg gcg gcc agc atg tat ccg gat cag Gln Val Ala Ala Asp Ala Asn Leu Ala Ala Ser Met Tyr Pro Asp Gln 95 100 105	1958
50	tcg ctc tat ccg gca aac tcg gtg cca gct gtg gtg gag cgg atc aac Ser Leu Tyr Pro Ala Asn Ser Val Pro Ala Val Val Glu Arg Ile Asn 110 115 120 125	2006
55	aac acc ttc cgt cgt gcc gat cag atc caa tgg tcc gcg ggc att gag Asn Thr Phe Arg Arg Ala Asp Gln Ile Gln Trp Ser Ala Gly Ile Glu 130 135 140	2054
60	ccg ggc gat ccg cgc tat gtc gat tac ttc ctg ccg atc gtt gcc gat Pro Gly Asp Pro Arg Tyr Val Asp Tyr Phe Leu Pro Ile Val Ala Asp 145 150 155	2102
65	gcg gaa gcc ggt ttt ggc ggt gtc ctg aat gcc ttt gaa ctg atg aaa Ala Glu Ala Gly Phe Gly Gly Val Leu Asn Ala Phe Glu Leu Met Lys 160 165 170	2150
70	gcg atg att gaa gcc ggt gca gcg gca gtt cac ttc gaa gat cag ctg Ala Met Ile Glu Ala Gly Ala Ala Ala Val His Phe Glu Asp Gln Leu 175 180 185	2198
75	gcg tca gtg aag aaa tgc ggt cac atg ggc ggc aaa gtt tta gtg cca Glu Thr Val Ala Asp Gln Gly Ala Thr Ser Glu Leu Val Leu Val Leu 190 195 200 205	2246

	act cag gaa gct att cag aaa ctg gtc gcg gcg cgt ctg gca gct gac	2294
	Thr Gln Glu Ala Ile Gln Lys Leu Val Ala Ala Arg Leu Ala Ala Asp	
	210 215 220	
5	gtg acg ggc gtt cca acc ctg ctg gtt gcc cgt acc gat gct gat gcg	2342
	Val Thr Gly Val Pro Thr Leu Leu Val Ala Arg Thr Asp Ala Asp Ala	
	225 230 235	
10	gcg gat ctg atc acc tcc gat tgc gac ccg tat gac agc gaa ttt att	2390
	Ala Asp Leu Ile Thr Ser Asp Cys Asp Pro Tyr Asp Ser Glu Phe Ile	
	240 245 250	
15	acc ggc gag cgt acc agt gaa ggc ttc ttc cgt act cat gcg ggc att	2438
	Thr Gly Glu Arg Thr Ser Glu Gly Phe Phe Arg Thr His Ala Gly Ile	
	255 260 265	
20	gag caa gcg atc agc cgt ggc ctg gcg tat gcg cca tat gct gac ctg	2486
	Glu Gln Ala Ile Ser Arg Gly Leu Ala Tyr Ala Pro Tyr Ala Asp Leu	
	270 275 280 285	
	gtc tgg tgt gaa acc tcc acg ccg gat ctg gaa ctg gcg cgt cgc ttt	2534
	Val Trp Cys Glu Thr Ser Thr Pro Asp Leu Glu Leu Ala Arg Arg Phe	
	290 295 300	
25	gca caa gct atc cac gcg aaa tat ccg ggc aaa ctg ctg gct tat aac	2582
	Ala Gln Ala Ile His Ala Lys Tyr Pro Gly Lys Leu Leu Ala Tyr Asn	
	305 310 315	
30	tgc tcg ccg tcg ttc aac tgg cag aaa aac ctc gac gac aaa act att	2630
	Cys Ser Pro Ser Phe Asn Trp Gln Lys Asn Leu Asp Asp Lys Thr Ile	
	320 325 330	
35	gcc agc ttc cag cag cag ctg tcg gat atg ggc tac aag ttc cag ttc	2678
	Ala Ser Phe Gln Gln Gln Leu Ser Asp Met Gly Tyr Lys Phe Gln Phe	
	335 340 345	
40	atc acc ctg gca ggt atc cac agc atg tgg ttc aac atg ttt gac ctg	2726
	Ile Thr Leu Ala Gly Ile His Ser Met Trp Phe Asn Met Phe Asp Leu	
	350 355 360 365	
	gca aac gcc tat gcc cag ggc gag ggt atg aag cac tac gtt gag aaa	2774
	Ala Asn Ala Tyr Ala Gln Gly Glu Gly Met Lys His Tyr Val Glu Lys	
	370 375 380	
45	gtg cag cag ccg gaa ttt gcc gcc gcg aaa gat ggc tat acc ttc gta	2822
	Val Gln Gln Pro Glu Phe Ala Ala Ala Lys Asp Gly Tyr Thr Phe Val	
	385 390 395	
50	tct cac cag cag gaa gtg ggt aca ggt tac ttc gat aaa gtg acg act	2870
	Ser His Gln Gln Glu Val Gly Thr Gly Tyr Phe Asp Lys Val Thr Thr	
	400 405 410	
55	att att cag ggc ggc acg tct tca gtc acc gcg ctg acc ggc tcc act	2918
	Ile Ile Gln Gly Gly Thr Ser Val Thr Ala Leu Thr Gly Ser Thr	
	415 420 425	
60	gaa gaa tcg cag ttc taa gcaacaacaa ccgttgctga ctgtaggccg	2966
	Glu Glu Ser Gln Phe	
	430	
	gataaggcgt tcacgccgca tccggcaatc ggtgcacgat gectgatgcg acgcttgccg	3026
	gtcttatcat gectacagcc gttgccgaac gtaggctgga taaggcgttt acgcgcgcatc	3086
	440 445 450 455 460	

ctcaaaccat ttgcaaggc ttcgatgctc agtatggctc attcctcgaa gtgacctccg 3206
 5 gtgcgcagca gcgtttcgaa caggccgact ggcattgctgt ccagcaggcg atgaaaaaac 3266
 gtatccatct ttacgatcat cacgttggtc tggtcgtgga gcaactgcgc tgcattacta 3326
 acggccaaag tacggacgcg gcatttttac tacgtgttaa agagcattac acccggtgt 3386
 10 tgccggatta cccgcgcttc gagattgcgg agagcttttt taactccgtg tactgtcgg 3446
 tatttgacca ccgctcgctt actcccgagc ggctttt0tat ctttagctct cagccagagc 3506
 15 gccgctttcg taccattccc cggccgctgg cgaaagactt tcaccccgat cacggctggg 3566
 aatctctact gatgcgcgtt atcagcgacc taccgctgcg cctgcgctgg cagaataaaa 3626
 gccgtgacat ccattacatt attcgccatc tgacggaaac gctggggaca gacaacctcg 3686
 20 cgaaagtca ttacaggtg gcgaacgaac tgttttaccg caataaagcc gcctggctgg 3746
 taggcaaac gatcacacct tccggcacat tgccattttt gctgccgatc caccagacgg 3806
 25 acgacggcga gttatttatt gatacctgcc tgacgacgac cgccgaagcg agcattgttt 3866
 ttggctttgc gcgttcttat tttatggttt atgcgcgcgt gccgcgagcg ctggctcag 3926
 ggctacggga aattctgcca ggtaaaacca ccgctgaatt gtatatggct atcggtgcc 3986
 30 agaagcacgc caaaaccgaa agctaccgcg aatatctcgt ttatctacag ggctgtaatg 4046
 agcagttcat tgaagcgccg ggtattcgtg gaatgggtgat gttgggtgtt acgctgccg 4106
 35 gctttgatcg ggtattcaaa gtcacaaag acaggttcgc gccgcagaaa gagatgtctg 4166
 ccgctcacgt tcgtgcctgc tatcaactgg tgaagagca cgatcgctg ggccgaatgg 4226
 cggacacca ggagtttgaa aactttgtgc tggagaagcg gcatatttcc ccggcattaa 4286
 40 tgggaattact gcttcaggaa gcagcggaaa aaatcaccga tctcgcgaa caaattgtga 4346
 ttcgccatct ttatattgag cggcggtatg tgccgctcaa tatctggctg gaacaagtgg 4406
 45 aaggtcagca gttgcgcgac gccattgaag aatacggtaa cgctattcgc cagcttgccg 4466
 ctgctaacat tttccctggc gacatgctgt ttaaaaactt cgggtgtcacc cgtcacgggc 4526
 gtgtgggttt ttatgattac gatgaaattt gctacatgac ggaagtgaat ttccgcgaca 4586
 50 tcccgcgcgc gcgctatccg gaagacgaac ttgccagcga accgtggtac agcgtctcgc 4646
 cgggcgatgt tttcccgga gagtttcgcc actggctatg cgccgacccg cgtattggct 4706
 cgctgtttga agagatgcac gccgacctgt tccgcgctga ttactggcgc gcactacaaa 4766
 55 accgcatacg tgaaggcat gtggaagatg tttatgcgta tcggcgagc caaagattta 4826
 gcgtacggta tggggagatg cttttttga 4855
 60 <210> 2
 <211> 434
 <212> PRT
 <213> Escherichia coli

<400> 2
 Met Lys Thr Arg Thr Gln Gln Ile Glu Glu Leu Gln Lys Glu Trp Thr
 1 5 10 15
 5 Gln Pro Arg Trp Glu Gly Ile Thr Arg Pro Tyr Ser Ala Glu Asp Val
 20 25 30
 Val Lys Leu Arg Gly Ser Val Asn Pro Glu Cys Thr Leu Ala Gln Leu
 35 40 45
 10 Gly Ala Ala Lys Met Trp Arg Leu Leu His Gly Glu Ser Lys Lys Gly
 50 55 60
 Tyr Ile Asn Ser Leu Gly Ala Leu Thr Gly Gly Gln Ala Leu Gln Gln
 65 70 75 80
 15 Ala Lys Ala Gly Ile Glu Ala Val Tyr Leu Ser Gly Trp Gln Val Ala
 85 90 95
 20 Ala Asp Ala Asn Leu Ala Ala Ser Met Tyr Pro Asp Gln Ser Leu Tyr
 100 105 110
 Pro Ala Asn Ser Val Pro Ala Val Val Glu Arg Ile Asn Asn Thr Phe
 115 120 125
 25 Arg Arg Ala Asp Gln Ile Gln Trp Ser Ala Gly Ile Glu Pro Gly Asp
 130 135 140
 Pro Arg Tyr Val Asp Tyr Phe Leu Pro Ile Val Ala Asp Ala Glu Ala
 145 150 155 160
 Gly Phe Gly Gly Val Leu Asn Ala Phe Glu Leu Met Lys Ala Met Ile
 165 170 175
 35 Glu Ala Gly Ala Ala Ala Val His Phe Glu Asp Gln Leu Ala Ser Val
 180 185 190
 Lys Lys Cys Gly His Met Gly Gly Lys Val Leu Val Pro Thr Gln Glu
 195 200 205
 40 Ala Ile Gln Lys Leu Val Ala Ala Arg Leu Ala Ala Asp Val Thr Gly
 210 215 220
 Val Pro Thr Leu Leu Val Ala Arg Thr Asp Ala Asp Ala Ala Asp Leu
 225 230 235 240
 Ile Thr Ser Asp Cys Asp Pro Tyr Asp Ser Glu Phe Ile Thr Gly Glu
 245 250 255
 50 Arg Thr Ser Glu Gly Phe Phe Arg Thr His Ala Gly Ile Glu Gln Ala
 260 265 270
 Ile Ser Arg Gly Leu Ala Tyr Ala Pro Tyr Ala Asp Leu Val Trp Cys
 275 280 285
 55 Glu Thr Ser Thr Pro Asp Leu Glu Leu Ala Arg Arg Phe Ala Gln Ala
 290 295 300
 Ile His Ala Lys Tyr Pro Gly Lys Leu Leu Ala Tyr Asn Cys Ser Pro
 305 310 315 320
 Ser Phe Asn Trp Gln Lys Asn Leu Asp Asp Lys Thr Ile Ala Ser Phe
 325 330 335
 60 Gln Gln Gln Leu Ser Asp Met Gly Gly Lys Ser Gln Phe Ile Thr Leu

	340	345	350	
	Ala Gly Ile His Ser Met Trp Phe Asn Met Phe Asp Leu Ala Asn Ala			
	355	360	365	
5	Tyr Ala Gln Gly Glu Gly Met Lys His Tyr Val Glu Lys Val Gln Gln			
	370	375	380	
10	Pro Glu Phe Ala Ala Ala Lys Asp Gly Tyr Thr Phe Val Ser His Gln			
	385	390	395	400
	Gln Glu Val Gly Thr Gly Tyr Phe Asp Lys Val Thr Thr Ile Ile Gln			
	405	410	415	
15	Gly Gly Thr Ser Ser Val Thr Ala Leu Thr Gly Ser Thr Glu Glu Ser			
	420	425	430	
	Gln Phe			
20	<210> 3			
	<211> 20			
	<212> DNA			
	<213> Artificial sequence			
25	<220>			
	<221> Primer			
	<222> (1)..(20)			
	<223> aceA `5`-1			
30	<400> 3			
	atgcttactc acgcctgttg			20
35	<210> 4			
	<211> 20			
	<212> DNA			
	<213> Artificial sequence			
40	<220>			
	<221> Primer			
	<222> (1)..(20)			
	<223> aceA `5`-2			
45	<400> 4			
	catgtgcaga tgctccatag			20
50	<210> 5			
	<211> 20			
	<212> DNA			
	<213> Artificial sequence			
55	<220>			
	<221> Primer			
	<222> (1)..(20)			
	<223> aceA `3`-1			
60	<400> 5			
	caacaacaac cggttgctgac			20
65	<210> 6			
	<211> 20			
	<212> DNA			
	<213> Artificial sequence			

<220>
 <221> Primer
 <222> (1)..(20)
 <223> aceA `3`-2
 5
 <400> 6
 cagttcgttc gccacctgta 20
 <210> 7
 <211> 1643
 <212> DNA
 <213> Escherichia coli
 10
 <220>
 <221> misc_feature
 <222> (1)..(33)
 <223> Technical DNA/ remainder polylinker sequence
 15
 <220>
 <221> misc_feature
 <222> (34)..(742)
 <223> Part of the 3' region of the aceB gene, intergenic region,
 ATG start codon of the aceA gene
 20
 <220>
 <221> misc_feature
 <222> (743)..(810)
 <223> Technical DNA/ remainder polylinker sequence
 25
 <220>
 <221> misc_feature
 <222> (811)..(1591)
 <223> Part of the 5' region of the aceK gene
 30
 <220>
 <221> misc_feature
 <222> (1592)..(1643)
 <223> Technical DNA/ remainder polylinker sequence
 35
 <400> 7
 agcttggtac cgagatctgc agaattcgcc cttatgctta ctcacgcctg ttgattaaaa 60
 cctgccataa acgcggtgct tttgcgatgg gcggcatggc ggcgtttatt ccgagcaaag 120
 45 atgaagagca caataaccag gtgctcaaca aagtaaaagc ggataaatcg ctggaagcca 180
 ataacggtca cgatggcaca tggatcgctc acccaggcct tgcggacacg gcaatggcgg 240
 50 tattcaacga cattctcggc tcccgtaaaa atcagcttga agtgatgcgc gaacaagacg 300
 cgccgattac tgccgatcag ctgctggcac cttgtgatgg tgaacgcacc gaagaaggta 360
 tgccgcgcaa cattcgcgtg gctgtgcagt acatcgaagc gtggatctct ggcaacggct 420
 55 gtgtgccgat ttatggcctg atggaagatg cggcgacggc tgaaatttcc cgtacctcga 480
 tctggcagtg gatccatcat caaaaaacgt tgagcaatgg caaacgggtg accaaagcct 540
 60 tggtccgcca gatgctgggc gaagagatga aagtcattgc cagcgaactg ggcaagaagc 600
 gtttctccca ggggcgtttt gacgatgccg cacgcttgat ggaacagatc accacttccg 660
 atgagtcacg cgatctcggc acccggcagc gctacggcct gctagcgtta accacacat 720
 65

	aactatggag catctgcaca tgaagggcga attccagcac actggcggcc gttactagta	780
	acggccgcca gtgtgctgga attcgccctt caacaacaac cgttgctgac tgtaggccgg	840
5	ataaggcggt cagccgcat cggcaatcg gtgcacgatg cctgatgca cgcttgcgcg	900
	tcttatcatg cctacagccg ttgccgaacg taggctggat aaggcgttta cgccgcatcc	960
10	ggcaattctc tgctcctgat gagggcgcta aatgccgctt ggcctggaat tattgattgc	1020
	tcaaaccatt ttgcaaggct tcgatgctca gtatggctga ttcctcgaag tgacctccgg	1080
	tgccgagcag cgtttcgaac agggcgactg gcatgctgtc cagcaggcga tgaaaaaccg	1140
15	tatccatctt tacgatcatc acgttggtct ggctgctggag caactgcgct gcattactaa	1200
	cggccaaaagt acggacgcgg cttttttact acgtgttaaa gagcattaca cccggctggt	1260
20	gccggattac ccgcgcttcg agattgcgga gagctttttt aactccgtgt actgtcggtt	1320
	atttgaccac cgctcgctta ctcccagcgc gctttttatc tttagctctc agccagagcg	1380
	ccgcttttcgt accattcccc gcccgctggc gaaagacttt caccgccatc acggctggga	1440
25	atctctactg atgcgcgtta tcagcgacct accgctgcgc ctgcgctggc agaataaaag	1500
	ccgtgacatc cattacatta ttcgccatct gacggaaaacg ctggggacag acaacctcgc	1560
30	ggaaagtcac ttacaggtgg cgaacgaact gaagggcgaa ttctgcagat atccatcaca	1620
	ctggcgggccg ctcgagcatg cat	1643

PCT

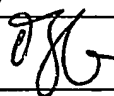
010131 BT

Original (for SUBMISSION) - printed on 05.03.2002 09:26:15 AM

0-1	Form - PCT/RO/134 (EASY) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)	
0-1-1	Prepared using	PCT-EASY Version 2.92 (updated 01.01.2002)
0-2	International Application No.	
0-3	Applicant's or agent's file reference	010131 BT

1	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
1-1	page	13
1-2	line	1-5
1-3	Identification of Deposit	
1-3-1	Name of depositary institution	DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
1-3-2	Address of depositary institution	Mascheroder Weg 1b, D-38124 Braunschweig, Germany
1-3-3	Date of deposit	08 September 2000 (08.09.2000)
1-3-4	Accession Number	DSMZ 13720
1-4	Additional Indications	NONE
1-5	Designated States for Which Indications are Made	all designated States
1-6	Separate Furnishing of Indications These indications will be submitted to the International Bureau later	NONE

FOR RECEIVING OFFICE USE ONLY

0-4	This form was received with the international application: (yes or no)	YES
0-4-1	Authorized officer	 B. GATINET (0)70/3402181

FOR INTERNATIONAL BUREAU USE ONLY

0-5	This form was received by the international Bureau on:	
0-5-1	Authorized officer	

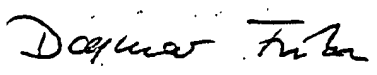
BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

PCT/EP02/02421

INTERNATIONAL FORM

Degussa-Hüls AG
Kantstr. 2
33790 Halle

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: DH5 α /pMAK705	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 13720
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I. above was accompanied by: <input checked="" type="checkbox"/> a scientific description <input checked="" type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable).	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2000-09-08 (Date of the original deposit) ¹ .	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  DSM 13720-13721

¹ Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE


PCT/EP02/02421

INTERNATIONAL FORM

Degussa-Hüls AG
Kantstr. 2
33790 Halle

VIABILITY STATEMENT

issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
Identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Degussa-Hüls AG Kantstr. 2 Address: 33790 Halle	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 13720 Date of the deposit or the transfer ¹ : 2000-09-08
III. VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on 2000-09-08 On that date, the said microorganism was <input checked="" type="checkbox"/> viable <input type="checkbox"/> no longer viable	
IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED²	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Date: 2000-09-12

- ¹ Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
- ² In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.
- ³ Mark with a cross the applicable box.
- ⁴ Fill in if the information has been requested.

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
17 October 2002 (17.10.2002)

PCT

(10) International Publication Number
WO 02/081722 A3

(51) International Patent Classification⁷: C12P 13/08,
13/04 // (C12P 13/08, C12R 1:19)

(21) International Application Number: PCT/EP02/02421

(22) International Filing Date: 6 March 2002 (06.03.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
101 16 518.8 3 April 2001 (03.04.2001) DE

(71) Applicant: DEGUSSA AG [DE/DE]; Bennigsenplatz 1,
40474 Düsseldorf (DE).

(72) Inventors: RIEPING, Mechthild; Mönkebergstrasse 1,
33619 Bielefeld (DE). HERMANN, Thomas; Zirkon-
strasse 8, 33739 Bielefeld (DE).

(81) Designated States (national): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,

GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG,
SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN,
YU, ZA, ZM, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR,
GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent
(BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR,
NE, SN, TD, TG).

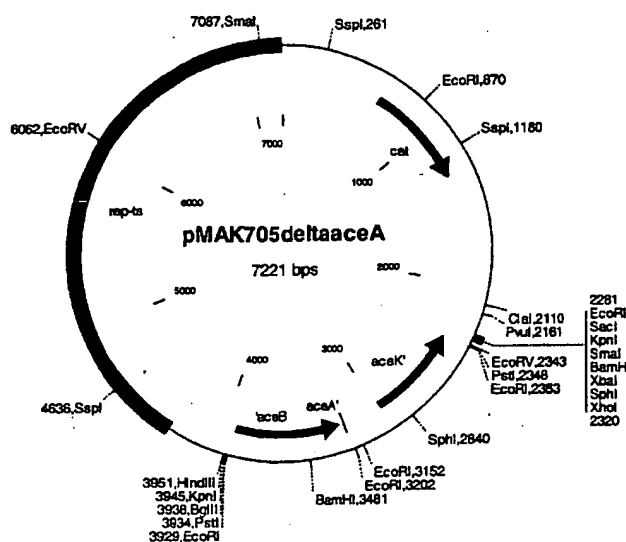
Published:

- with international search report
- with (an) indication(s) in relation to deposited biological
material furnished under Rule 13bis separately from the
description

(88) Date of publication of the international search report:
30 October 2003

For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: PROCESS FOR THE PRODUCTION OF L-AMINO ACIDS USING STRAINS OF THE FAMILY ENTEROBACTE-
RIACEAE THAT CONTAIN AN ATTENUATED ACEA GENE



(57) Abstract: The invention relates to a process for the production of L-amino acids, in particular L-threonine, in which the fol-
lowing steps are carried out: a) fermentation of the microorganisms of the family Enterobacteriaceae producing the desired L-amino
acid, in which the aceA gene or nucleotide sequences coding therefor are attenuated, in particular are switched off; b) enrichment of
the L-amino acid in the medium or in the cells of the bacteria; and c) isolation of the L-amino acid.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/02421

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12P13/08 C12P13/04 //(C12P13/08,C12R1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12P C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4 368 266 A (TOSAKA OSAMU ET AL) 11 January 1983 (1983-01-11) column 1, line 39 -column 2, line 8 examples 1-3 tables 3-5 claim 1	1-7
Y	EP 0 952 221 A (AJINOMOTO KK) 27 October 1999 (1999-10-27) page 2, line 24 -page 3, line 2 page 6, line 42 -page 7, line 6 claim 1	1-7
Y	EP 0 955 368 A (AJINOMOTO KK) 10 November 1999 (1999-11-10) page 2, line 22-56 page 6, line 9-31 claim 6	1-7
	-/-	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *Z* document member of the same patent family

Date of the actual completion of the international search

20 May 2003

Date of mailing of the international search report

02/06/2003

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

van de Kamp, M

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/02421

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MATSUOKA M ET AL.: "Isolation, hyperexpression, and sequencing of the aceA gene encoding isocitrate lyase in Escherichia coli" JOURNAL OF BACTERIOLOGY, vol. 170, no. 10, 1988, pages 4528-4536, XP008016923 ISSN: 0021-9193 cited in the application abstract	1-7
A	MICHAL G: "Biochemical pathways: an atlas of biochemistry and molecular biology" 1999, JOHN WILEY & SONS INC. AND SPEKTRUM AKADEMISCHER VERLAG, NEW YORK - HEIDELBERG XP002240819 ISBN: 0-471-33130-9 figures 3.8-2, 3.9-1 figures 4.2-1, 4.5-1 and 4.5-2	1-7
A	KRAEMER R: "Genetic and physiological approaches for the production of amino acids" JOURNAL OF BIOTECHNOLOGY, vol. 45, no. 1, 1996, pages 1-21, XP002178648 ISSN: 0168-1656 the whole document	1-7
A	US 4 278 765 A (DEBBOV VLADIMIR G ET AL) 14 July 1981 (1981-07-14) cited in the application the whole document	1-7
A	EP 0 643 135 A (AJINOMOTO KK) 15 March 1995 (1995-03-15) the whole document	1-7
A	EP 0 237 819 A (KYOWA HAKKO KOGYO KK) 23 September 1987 (1987-09-23) the whole document	1-7
A	DATABASE WPI Section Ch, Week 199148 Derwent Publications Ltd., London, GB; Class B05, AN 1991-351136 XP002241222 & JP 03 236786 A (KYOWA HAKKO KOGYO KK), 22 October 1991 (1991-10-22) abstract	1-7

-/--

INTERNATIONAL SEARCH REPORT

Intern. Patent Application No
PCT/EP 02/02421

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 99 53035 A (ALTMAN ELLIOT ;GOKARN RAVI R (US); EITEMAN MARK A (US); UNIV GEORG) 21 October 1999 (1999-10-21) page 5, line 20-24 examples 4,7,9,10 claims 41,49 figures 1,4 ---	1-7
A	JETTEN M S M ET AL.: "Recent advances in the physiology and genetics of amino acid-producing bacteria." CRC CRITICAL REVIEWS IN BIOTECHNOLOGY, vol. 15, no. 1, 1995, pages 73-103, XP000613291 ISSN: 0738-8551 figure 1 page 83, right-hand column, line 1-36 page 90, left-hand column, line 1 -page 92, left-hand column, line 17 ---	1-7
A	CHUNG T ET AL.: "Glyoxylate bypass operon of Escherichia coli: cloning and determination of the functional map." JOURNAL OF BACTERIOLOGY, vol. 170, no. 1, January 1988 (1988-01), pages 386-392, XP008015355 ISSN: 0021-9193 abstract ---	1-7
A	SAWERS G: "The anaerobic degradation of L-serine and L-threonine in enterobacteria: networks of pathways and regulatory signals" ARCHIVES OF MICROBIOLOGY, vol. 171, no. 1, 1998, pages 1-5, XP002953871 ISSN: 0302-8933 the whole document ---	1-7
E	WO 02 081698 A (DEGUSSA) 17 October 2002 (2002-10-17) the whole document page 9, line 21 -page 10, line 22 claim 7 ---	1-7
E	WO 02 081721 A (DEGUSSA) 17 October 2002 (2002-10-17) the whole document page 9, line 24 -page 10, line 25 claim 7 -----	1-7

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 02/02421

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
US 4368266	A	11-01-1983	JP 1426831 C	25-02-1988
			JP 57018990 A	30-01-1982
			JP 62036677 B	07-08-1987
			JP 1426801 C	25-02-1988
			JP 56092794 A	27-07-1981
			JP 62036675 B	07-08-1987
			FR 2472610 A1	03-07-1981
EP 0952221	A	27-10-1999	AU 756507 B2	16-01-2003
			AU 2122399 A	30-09-1999
			BR 9901173 A	28-03-2000
			CN 1233660 A	03-11-1999
			EP 0952221 A2	27-10-1999
			JP 2000189169 A	11-07-2000
			PL 332072 A1	27-09-1999
			US 6331419 B1	18-12-2001
			US 2001019836 A1	06-09-2001
EP 0955368	A	10-11-1999	AU 746542 B2	02-05-2002
			AU 2122499 A	30-09-1999
			BR 9901174 A	28-03-2000
			CN 1233661 A	03-11-1999
			EP 0955368 A2	10-11-1999
			JP 2000106869 A	18-04-2000
			PL 332071 A1	27-09-1999
			RU 2188236 C2	27-08-2002
			US 6197559 B1	06-03-2001
			US 2002004231 A1	10-01-2002
US 4278765	A	14-07-1981	SU 875663 A1	15-09-1982
			HU 190999 B	28-12-1986
EP 0643135	A	15-03-1995	AT 203769 T	15-08-2001
			CZ 9401658 A3	15-12-1994
			DE 69330518 D1	06-09-2001
			DE 69330518 T2	08-05-2002
			DK 643135 T3	15-10-2001
			EP 0643135 A1	15-03-1995
			JP 3331472 B2	07-10-2002
			SK 81994 A3	10-05-1995
			US 5661012 A	26-08-1997
			EP 1020526 A2	19-07-2000
			ES 2158867 T3	16-09-2001
			WO 9411517 A1	26-05-1994
			RU 2113484 C1	20-06-1998
EP 0237819	A	23-09-1987	DE 3788583 D1	10-02-1994
			DE 3788583 T2	19-05-1994
			EP 0237819 A2	23-09-1987
			JP 2574786 B2	22-01-1997
			JP 63273487 A	10-11-1988
			KR 9108634 B1	19-10-1991
			US 5017483 A	21-05-1991
JP 3236786	A	22-10-1991	JP 2877414 B2	31-03-1999
WO 9953035	A	21-10-1999	AU 3555999 A	01-11-1999
			BR 9909615 A	12-12-2000

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 02/02421

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9953035 A		CA 2325598 A1	21-10-1999
		EP 1073722 A1	07-02-2001
		JP 2002511250 T	16-04-2002
		WO 9953035 A1	21-10-1999
		US 2003087381 A1	08-05-2003
		US 6455284 B1	24-09-2002
WO 02081698 A	17-10-2002	DE 10116518 A1	17-10-2002
		WO 02081721 A2	17-10-2002
		WO 02081698 A2	17-10-2002
		WO 02081722 A2	17-10-2002
		US 2003054503 A1	20-03-2003
		US 2003059903 A1	27-03-2003
WO 02081721 A	17-10-2002	DE 10116518 A1	17-10-2002
		WO 02081721 A2	17-10-2002
		WO 02081698 A2	17-10-2002
		WO 02081722 A2	17-10-2002
		US 2003054503 A1	20-03-2003
		US 2003059903 A1	27-03-2003